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Journal

Journal of the American Chemical Society, 145(41)

ISSN

0002-7863

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Publication Date

2023-10-18

DOI

10.1021/jacs.3c07803

Peer reviewed



Published in final edited form as:

J Am Chem Soc. 2023 October 18; 145(41): 22305–22309. doi:10.1021/jacs.3c07803.

Detection of a Geminate Photoproduct of Bovine Cytochrome *c* Oxidase by Time-Resolved Serial Femtosecond Crystallography

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The manuscript was written through contributions of all authors.

The authors declare no competing financial interest.

Complete contact information is available at: <https://pubs.acs.org/10.1021/jacs.3c07803>

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.3c07803>.

Materials and methods, additional structural information, and crystallographic data collection and refinement statistics (PDF)

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Abstract

Cytochrome *c* oxidase (CcO) is a large membrane-bound heme protein that catalyzes the reduction of dioxygen to water. Unlike classical dioxygen binding heme proteins with a heme *b* group in their active sites, CcO has a unique binuclear center (BNC) composed of a copper atom (Cu_B) and a heme *a*₃ iron, where O₂ binds and is reduced to water. CO is a versatile O₂ surrogate in ligand binding and escape reactions. Previous time-resolved spectroscopic studies of the CO complexes of bovine CcO (bCcO) revealed that photolyzing CO from the heme *a*₃ iron leads to a metastable intermediate (Cu_B-CO), where CO is bound to Cu_B, before it escapes out of the BNC. Here, with a pump-probe based time-resolved serial femtosecond X-ray crystallography, we detected a geminate photoproduct of the bCcO-CO complex, where CO is dissociated from the heme *a*₃ iron and moved to a temporary binding site midway between the Cu_B and the heme *a*₃ iron, while the locations of the two metal centers and the conformation of Helix-X, housing the

proximal histidine ligand of the heme a_3 iron, remain in the CO complex state. This new structure, combined with other reported structures of bCcO, allows for a clearer definition of the ligand dissociation trajectory as well as the associated protein dynamics.

Cytochrome *c* oxidase (CcO) is the terminal enzyme in the electron transfer chain in the inner membrane of mitochondria. It reduces dioxygen to two water molecules by accepting four electrons from cytochrome *c* and four protons from the negative side of the mitochondrial membrane. At the same time, it harnesses the chemical energy derived from the dioxygen reduction chemistry to translocate four protons from the negative to positive side of the membrane for the production of the electrochemical proton gradient required for ATP synthesis by ATP synthase.¹ To accomplish this complex task, CcO possesses four redox active centers, Cu_A, heme *a*, and a binuclear center (BNC) formed by a copper atom (Cu_B) and the heme a_3 iron,² where O₂ binds and is reduced to water. The dioxygen reduction reaction catalyzed by CcO has been extensively studied and is well-understood.^{3–7} In contrast, the O₂ binding dynamics preceding the oxygen chemistry remains elusive as it is technically challenging to monitor the fleeting O₂ complex.

Carbon monoxide (CO), like O₂, is an excellent ligand for heme proteins, but unlike O₂, it forms nonreactive complexes with them. Furthermore, when CO is bound to a heme iron, it is readily dissociable by a short pulse of visible light, making it a versatile tool for the investigation of ligand dissociation and rebinding dynamics.^{8,9} Time-resolved Fourier-transform infrared spectroscopic studies at ambient temperatures showed that photolyzing CO from the heme a_3 iron in the BNC of bovine CcO (bCcO) in free solution leads to the formation of a characteristic Cu_B-CO intermediate (Figure 1A), where the photolyzed CO is coordinated to the Cu_B, in less than 1 ps.^{10–14} The photolyzed CO subsequently dissociates from Cu_B and escapes out of the BNC in $\sim 1.5 \mu\text{s}$ ^{11,15} to generate the ligand-free reduced species (R). By extension, it is believed that the bimolecular binding reaction of CO, as well as O₂, follows the same trajectory, but in a reverse order, with Cu_B as a temporary ligand stopping point.^{6,16–18} In addition to the Cu_B-CO intermediate, transient UV–vis absorption spectroscopic data suggest that a geminate photoproduct, where CO has been photolyzed from heme a_3 iron but not yet coordinated to Cu_B, is formed immediately following CO photodissociation.^{11,15} This proposed intermediate, however, has never been experimentally identified.

Crystallographic studies of the bCcO–CO complex showed that X-rays from synchrotron light sources, like visible light, can photolyze CO from heme a_3 .¹⁹ To prevent the X-ray induced radiation damage, we employed serial femtosecond crystallography (SFX)²⁰ to determine the structure of the intact bCcO–CO complex.¹⁹ With SFX, the diffraction patterns of randomly oriented microcrystals suspended in an aqueous jet, which intersects the femtosecond pulses from an X-ray free-electron laser (XFEL), are sequentially collected and then merged for structural determination. As the radiation damage processes do not occur until after the termination of each ultrashort laser pulse, radiation damage-free structures can be obtained under native-like conditions at ambient temperatures.²⁰ The comparison of the SFX structure of the bCcO–CO complex with that of the ligand-free protein (R) (Figure 1B) shows that CO dissociation leads to the displacement of the Cu_B-

H240 moiety toward the heme a_3 and the movement of the heme a_3 iron-H376 moiety away from the Cu_B center. The structural changes to the BNC are associated with a conformational transition in the [380–383] fragment of Helix-X (housing H376), from an open to a closed conformation,²¹ and a $\sim 120^\circ$ flip of the S382 side chain.

To comprehend the protein dynamics associated with the CO dissociation reaction, here we sought to employ a time-resolved SFX method, using an OPO laser as the pump beam (to photolyze CO from the bCcO-CO complex) and a XFEL as the probe beam to determine the structure of the photoproduct populated at 100 ns following the initiation of the reaction. The plate-like bCcO-CO microcrystals ($\sim 20 \times 20 \times 4 \mu\text{m}^3$ in size) were prepared with a previously reported protocol.¹⁹ A slurry of the microcrystals was loaded in a gastight syringe and injected into the XFEL beam as a thin ($4 \mu\text{m}$) solution jet in a vacuum chamber²² (see Material and Methods in the Supporting Information). Each OPO laser pulse (with a width of ~ 8 ns) was timed at 100 ns before the XFEL pulse (with a width of ~ 40 fs). The wavelength and pulse energy of the OPO laser were set at 492 nm and $60 \mu\text{J}$, respectively, to ensure that the CO was completely photolyzed. The serial diffraction patterns were collected for 85 min, among which 16,520 indexable patterns were merged and analyzed.

The initial structure was solved with molecular replacement using the structure of ligand-free reduced bCcO (PDB ID: 7THU) as the search model. In the F_O-F_C difference map contoured at 6σ (see inset-i in Figure 2A), clear ligand electron density is evident between Cu_B and heme a_3 ; in addition, there is no residual electron density connecting the ligand density to the heme a_3 iron (even when the map is contoured at 3σ), indicating that CO is completely photolyzed and that there is no geminate CO recombination (which is in good agreement with free solution reactions).²³ As the C and O atoms are indistinguishable in the current data, the ligand electron density was arbitrarily modeled with the atom closer to the iron assigned as the C atom (inset-ii). The final structure was refined to a resolution of 2.8 \AA (PDB ID: 8GBT, see Supporting Information Table S1).

The structural data reveal that upon photodissociation the CO rotates $\sim 90^\circ$ with respect to its center of mass, such that it lies parallel with the heme a_3 plane, midway between Cu_B and the heme a_3 iron (Figure 2A). It is stabilized by sandwiching between heme a_3 and Cu_B , as well as by H291, H240 and V243, via either van der Waals and/or electrostatic interactions (see Figure S1 in the Supporting Information). The Cu_B -H240 and heme a_3 iron-H376 moieties, as well as the conformation of Helix-X, remain unperturbed, indicating that the protein matrix does not relax to the ligand-free R state until later during the reaction.

The current structure is distinct from that of the photoproduct reported by Shimada et al. (PDB ID: 5X1B),²⁴ derived from a different pump-probe experiment, where CO is photolyzed from the heme a_3 iron and coordinated to Cu_B (Figure 2B). With respect to the CO complex, the Cu_B atom moved toward the heme a_3 by 0.5 \AA , while the heme a_3 iron atom moved away from the heme plane by 0.2 \AA , resulting in the contraction of the Cu_B -heme a_3 iron distance from 5.3 to 5.0 \AA , but Helix-X remained in the CO-complex (open) state. The structural features of the two photoproducts, combined with the reaction scheme illustrated in Figure 1A, suggest that the intermediate reported in this work is the long-suspected primary geminate photoproduct, while the Shimada structure is the Cu_B -CO

intermediate. It is important to note that the two photoproducts are distinct from that generated during X-ray diffraction data collection induced by constant synchrotron light illumination (PDB ID: 5WAU)¹⁹ (see Figure S2 in the Supporting Information), as the latter represents a unique photostationary state, instead of a true reaction intermediate.

The availability of the structures of the two reaction intermediates of bCcO, combined with the equilibrium structures of the CO-complex and ligand-free R state, allows us to define the ligand dissociation trajectory and the associated protein dynamics, as depicted in Figure 3. The heme a_3 iron-CO bond scission first leads to the rotation of the CO to a new orientation parallel with the heme a_3 plane (step 1, indicated by the green arrow in Figure 3A), while the Cu_B-H240 and heme a_3 iron-H376 moieties and Helix-X remain in the CO-complex (open) state. Subsequently, CO further rotates toward Cu_B to establish a coordination bond with it (step 2, blue arrows). It triggers the relaxation of the BNC to a ligand-free R-like conformation, resulting in a 0.3 Å contraction of the Cu_B-Fe distance, while the conformation of Helix-X remains unperturbed. Finally, CO dissociates from Cu_B and escapes out of the BNC (step 3, red arrows), which is accompanied by a 0.2 Å re-expansion of the Cu_B-Fe distance and a structural transition of Helix-X to the equilibrium R (closed) conformation, thereby completing the reaction.

It is important to note that in free solution reactions CO was able to migrate to the Cu_B site within 1 ps following the photolysis from heme a_3 .^{25,26} The current data indicate that the reaction taking place in the microcrystals is at least 4 orders of magnitude slower. It is in agreement with our previous data showing that the oxygen reaction catalyzed by bCcO is ~10,000-fold slower in crystalline states as compared to that in solution phases.²⁷ They indicate that the crystal lattice constrains certain protein motions required for the protein to cross the activation energy barriers and call for computational studies to delineate the structural factors underlying the activation processes.

The overall protein structural transition induced by CO dissociation in bCcO is comparable to that in myoglobin (Mb), a model system considered as the hydrogen atom of biology.²⁸ Mb is a small water-soluble protein that contains a heme b group embedded in the protein matrix. The heme iron is coordinated by a histidine residue (H93) from Helix-F on the proximal side (equivalent to H376 in Helix-X in bCcO), and a diatomic ligand, such as O₂ or CO, on the distal side, which is stabilized by another histidine residue (H64) from Helix-E (in place of the Cu_B moiety in bCcO). Photodissociation of CO from the Mb-CO complex leads to a geminate photoproduct, where CO rotates and translates to a nearby docking site, such that it lies parallel with the heme plane as in bCcO, within the distal heme pocket.²⁹ It is accompanied by the displacement of the heme iron out of the heme plane, as well as the concurrent movement of (i) the proximal H93 and the associated Helix-F away from the heme, and (ii) the distal H64 and the associated Helix-E toward the heme.^{30–32} These structural changes near the active site then propagate to the rest of the protein matrix through a global protein relaxation in a quake-like motion to establish the final equilibrium ligand-free structure.^{30–32}

Despite the similarities, the protein dynamics associated with CO dissociation in bCcO is much less cooperative compared to that in Mb. The dissociation of CO from the heme iron

in bCcO, like that in Mb, first leads to the formation of a geminate photoproduct, with CO bound to a docking site within the distal heme pocket, but it does not introduce any immediate structural changes to the heme or the protein matrix surrounding it. The presence of Cu_B in the BNC in bCcO reroutes the CO migration pathway, rendering an additional Cu_B-CO intermediate. It is not until the CO dissociates from Cu_B that the proximal Helix-X relaxes to the final equilibrium in the R state.

The unique CO dissociation-induced protein dynamics in bCcO is accompanied by transient contraction of the BNC (see Figure 3B), which plausibly plays an important role in guiding the unidirectional movement of the ligand out of the BNC, thereby accounting for the lack of geminate recombination in the reaction, in contrast to the ~40% geminate recombination in the Mb reaction.³⁰ This characteristic protein plasticity in bCcO is conceivably critical for coupling the oxygen chemistry occurring in the BNC to proton translocation.

In summary, the data presented here provide the first direct evidence of the geminate photoproduct of bCcO. The definition of its structure, combined with a previously reported Cu_B-CO structure (PDB ID: 5X1B),²⁴ enables us to define the ligand dissociation trajectory, as well as the associated protein dynamics, more clearly for the first time. It offers exciting experimental blueprints for the computational interrogation of the energy landscape associated with the ligand migration reaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The SFX experiments were carried out at the Linac Coherent Light Source (LCLS) at the SLAC National Accelerator Laboratory. LCLS is an Office of Science User Facility operated for the US Department of Energy Office of Science by Stanford University. Use of the LCLS, SLAC National Accelerator Laboratory, is supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract DE-AC02-76SF00515. Parts of the sample delivery system used at LCLS for this research were funded by NIH Grant P41GM103393, formerly P41RR001209. This work was supported by National Science Foundation (NSF) grants CHE-1404929 (to D.L.R. and S.-R.Y.), ABI Innovations Award 1565180 (to N.A.Z.) and Science and Technology Center Award 1231306. This work was also supported by National Institutes of Health (NIH) grants GM126297 (D.L.R. and S.-R.Y.), GM115773 (S.-R.Y.), GM095583 (to P.F.), S10 OD023453 and P41 GM139687, as well as the Biodesign Center for Applied Structural Discovery at Arizona State University.

ABBREVIATIONS

CcO	cytochrome <i>c</i> oxidase
bCcO	bovine cytochrome <i>c</i> oxidase
Mb	myoglobin
BNC	binuclear center
CO	carbon monoxide
SFX	serial femtosecond crystallography

GDVN	gas dynamic virtual nozzle
LCLS	Linac Coherent Light Source

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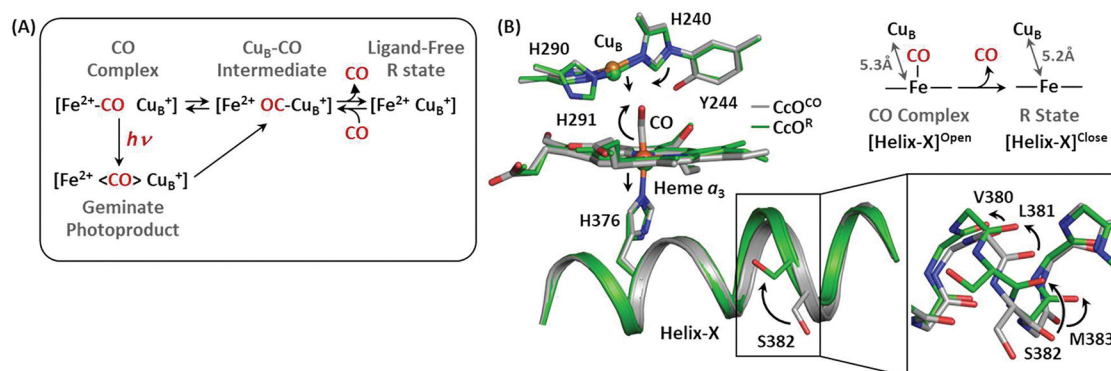


Figure 1. Proposed CO dissociation mechanism of bCcO (A) and the associated structural transition (B). The conformational changes induced by dissociation of CO from the CO complex (PDB ID: 5W97, gray) to generate the ligand-free R species (PDB ID: 7THU, green) are indicated by the arrows. The lower right inset shows the expanded view of the [380–383] fragment; the arrows indicate the rotation of the backbone carbonyl groups. The side chains, except that of S382, are not shown for clarity.

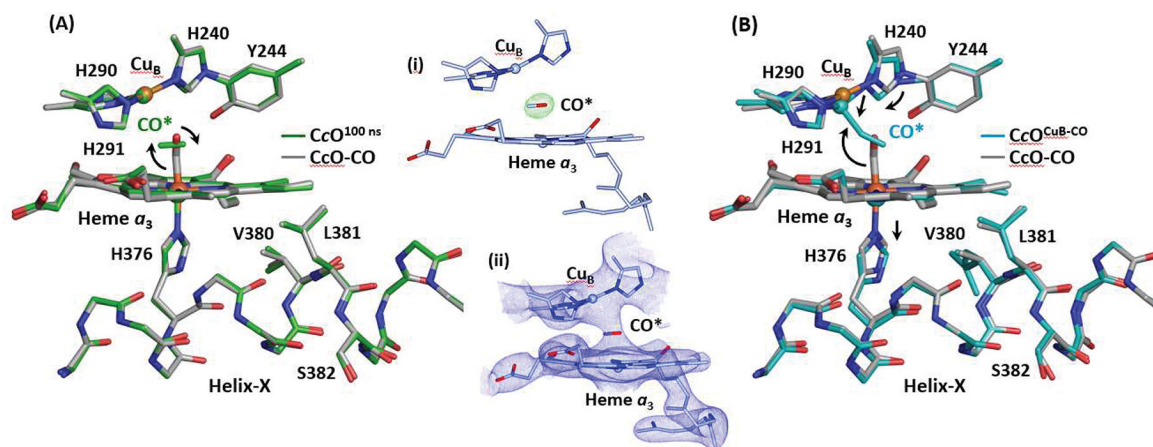


Figure 2. Structure of the geminate photoproduct of bCcO determined in this work (PDB ID: 8GBT, green) (A) with respect to the reported Cu_B-CO intermediate structure (PDB ID: 5X1B, cyan) (B). The structure of the CO complex (PDB ID: 5W97, gray) was shown as a reference in each panel to illustrate the CO dissociation induced structural changes, as indicated by the arrows. Inset (i) and (ii) show the F_O-F_C difference map and the 2F_O-F_C map (with CO modeled in) of the BNC contoured at 6 and 2 σ , respectively.

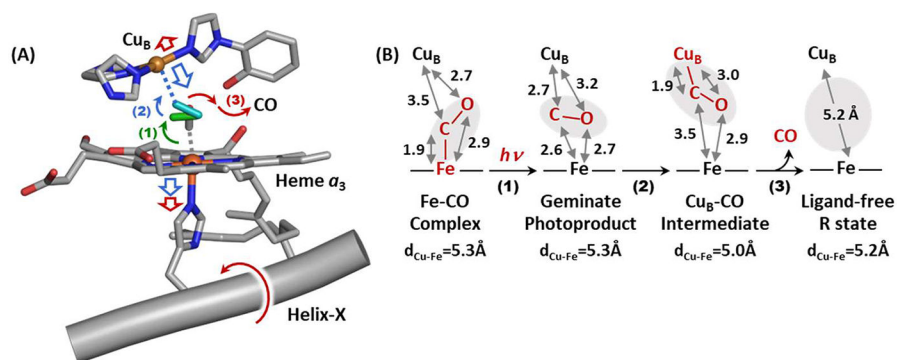


Figure 3. CO dissociation reaction trajectory in bCcO. (A) Schematic illustration of the 3-step reaction and (B) changes in the critical interatomic distances during the reaction.